Journal of Medical Virology 52:243–252 (1997)

Detection of Archetype and Rearranged Variants of JC Virus in Multiple Tissues From a Pediatric PML Patient

Jason T. Newman and Richard J. Frisque*

Department of Biochemistry and Molecular Biology, Pennsylvania State University, University Park, Pennsylvania

JC virus (JCV) establishes persistent infections in its human host, and in some immunocompromised individuals, the virus causes the fatal brain disease progressive multifocal leukoencephalopathy (PML). Two forms of the virus, archetype and rearranged, have been isolated, with the latter being derived from the archetype form by deletion and duplication of sequences within the viral transcriptional control region (TCR). We have used the PCR technique to amplify JCV TCR sequences present within multiple tissues of a pediatric PML patient and have cloned and sequenced the PCR products. Archetype JCV was readily detected in kidney tissue; this form of JCV was also identified for the first time in brain and lymph node tissue by employing archetype–specific PCR primers. In addition, several archetype–like variants containing small deletions within their regulatory regions were isolated from cardiac muscle and lung. Different, but related rearranged forms were detected in most of the tissues examined. Each of the rearranged TCRs lacked portions of a 66 base pair (bp) region found within the archetype promoter–enhancer but retained a 23 bp region that is deleted in the prototype (Mad 1) rearranged form of JCV. Although several rearranged forms of JCV were identified in this patient, the TCRs could be assigned to one of two groups based upon the deletion boundaries generated during the adaptation from archetype to rearranged JCV. This study is the first to characterize multiple JCV variants present in different tissues from a patient likely to have succumbed to PML during a primary infection. ***J. Med. Virol. 52:243–252, 1997.***

KEY WORDS: polyomavirus; immunodeficiency; PCR; transcriptional control region

INTRODUCTION

The human polyomavirus, JC virus (JCV), was first isolated from the brain of a patient with progressive multifocal leukoencephalopathy (PML) [Padgett et al., 1971]. Most of the world's population is exposed to this virus prior to reaching adulthood [Padgett and Walker, 1973; Taguchi et al., 1982; Walker and Padgett, 1983], and the usual outcome is the establishment of a persistent, asymptomatic infection. This innocuous relationship may be disrupted by a severe immunocompromising condition, leading to a reactivation of the infection and possible progression to PML [Frisque and White, 1992; Tornatore et al., 1994]. Whereas PML was once considered a rare disease that occurred in cancer and organ transplant patients, it is now recognized as the cause of death for up to 5% of AIDS patients [Berger et al., 1987; Kure et al., 1991].

JCV has been isolated from individuals with and without PML, and the virus can be classified as either an archetype or a rearranged form based upon the structure of the non-coding transcriptional control region (TCR). The archetypal TCR contains a single copy of the promoter-enhancer (Fig. 1). Archetype JCV is frequently detected in the urine and kidneys of immunocompetent as well as immunosuppressed individuals [Flægstad et al., 1991; Guo et al., 1996; Kitamura et al., 1994a; Loeber and Dörries, 1988; Markowitz et al., 1991; Tominaga et al., 1992; Yogo et al., 1990, 1991a], and it has been suggested that this is the form of JCV which circulates throughout the human population [Ault and Stoner, 1993; Flægstad et al., 1991; Yogo et al., 1991a]. The rearranged form of JCV represents a collection of viral variants which appear to arise in an individual through deletion and duplication of sequences within the archetype TCR [Ault and Stoner,

Contract grant sponsor: National Cancer Institute; Contract grant number: CA44970.

*Correspondence to: Richard J. Frisque, Department of Biochemistry and Molecular Biology, Pennsylvania State University, University Park, PA 16802.

Accepted 7 March 1997

TABLE I. Nucleotide Sequence of PCR Primers, Probes, and Sequencing Primers

Primer/probe name	Sequence 5'→3'	Nucleotide positions
JRR1	CCTCCACGCCCTTACTACTTCTGAG	5086–5110[a]
JRR1.1	CCTAGGGAGCCAACCAGCTAACAGC	164–188[a]
JRR2	GTGACAGCTGGCGAAGAACCATGGC	298–274[a]
JRR3	CCATGGATTCCTCCCTATTCAGCA	4980–5004[a]
JRR3.1	GCCAGCCAAGCATGAGCTCATACCT	142–166[a]
Arche#1	ACTTGTGATTAAGGACTATGGGAGGG	183–158[b]
Arche#2	CTGGCAGTTATAGTGAAACCCCTCCC	139–164[b]
M6R#1	GAGTAAGCTTGGAGG	5108–5122[a]
M6R#2	CAGCTGGTGACAAGC	273–259[a]
PCRII#1	GATCCACTAGTAACG	253–267[c]
PCRII#2	GTGATGGATATCTGC	304–290[c]

[a]Nucleotide numbering for JCV (Mad 1) is from Frisque et al. [1984].
[b]Nucleotide numbering for archetype JCV is from Yogo et al. [1990].
[c]Nucleotide numbering for vector pCR™2.1 is from the Original TA Cloning Kit Manual (Invitrogen).

1993; Flægstad et al., 1991; Tominaga et al., 1992; White et al., 1992; Yogo et al., 1990, 1991a]. It has been proposed that these rearrangements might generate variants with altered tissue tropism and pathogenic behavior [Loeber and Dörries, 1988; Martin et al., 1985]. The two forms do display distinct biological characteristics. Recently, it has been shown that rearranged JCV replicates more efficiently than archetype in cultured cells [Daniel et al., 1996]. Furthermore, in vivo studies have suggested that archetype JCV is confined to the kidneys, whereas rearranged forms are found in lymphocytes, brain, and kidneys [Ault and Stoner, 1993, 1994; Dörries et al., 1994; Kato et al., 1994; Loeber and Dörries, 1988; Martin et al., 1985; Tornatore et al., 1992; White et al., 1992; Yogo et al., 1994].

Prior to the AIDS epidemic, which has reduced the average age of onset to less than 40 years [Stoner et al., 1988], most cases of PML occurred in the elderly. In the adult patient, the disease is thought to arise following reactivation of a persistent infection. However, there have been a few reports of PML occurring in children with inherited immunodeficiency syndromes (e.g., SCID, Wiscott–Aldrich syndrome [Grinnell et al., 1983; Katz et al., 1994; Redfearn et al., 1993; Zu Rhein et al., 1978]) and AIDS [Berger et al., 1992]. It is possible that in these patients, a primary infection led directly to the disease. In one such case involving a 5-year-old child, Grinnell and co-workers [1983] detected non-integrated viral DNA in liver, lung, lymph node, and spleen using Southern hybridization. No attempt was made to isolate or characterize the JCV sequences being detected. With the development of PCR technology, however such a characterization became feasible. In the present study, we have examined the distribution and identity of JCV within this pediatric PML patient, and we discuss the relevance of these findings to the pathogenic potential of this opportunistic human virus.

MATERIALS AND METHODS

Human Tissue Samples

The PML tissues analyzed in this study were obtained from a 5-year-old child with severe combined immune deficiency syndrome (SCID) [Grinnell et al., 1983]. Specimens were received from Dr. Duard Walker (University of Wisconsin) as solid blocks of tissue, except for brain which was obtained as a 10% extract in phosphate buffered saline. Tissues included brain (Br), coeliac plexus (Cp), kidney (Kd), spleen (Sp), liver (Lv), lymph node (Ln), lung (Lu), and cardiac muscle (Cm). All samples were stored in individual vials and kept frozen at −80°C until use. Prior to the preparation of tissues, all working surfaces and tools were treated with 10% bleach and exposed to UV light for 30 min. Every effort was made to use disposable supplies (e.g., forceps, scalpels, and petri dishes), and each tissue was handled separately.

Tissue fragments (8 mm^3) were digested in microfuge tubes with 105 μl of tissue digestion buffer [White et al., 1992]. Samples were vortexed vigorously, incubated at 56°C for 90 min, and then transferred to a water bath or heat block at 95–100°C for 15 min to inactivate the proteinase K. The digested tissues were centrifuged, and the supernatants were stored at −80°C until subjected to PCR analysis. For the homogenized brain extract, a 10 μl aliquot was heated to 95–100°C for 15 min, and a 2 μl aliquot of the heat-treated sample was used in the PCR reaction.

PCR Precautions

The potential for contamination associated with any PCR reaction has been discussed at length. To minimize this risk, we adopted a number of recommended procedures [Jackson et al., 1991; Kwok, 1990; Prince and Andrus, 1992; White et al., 1992], including the physical separation of individual steps of the PCR procedure, utilization of single use aliquots of reagents, treatment of instruments with 10% bleach and/or UV

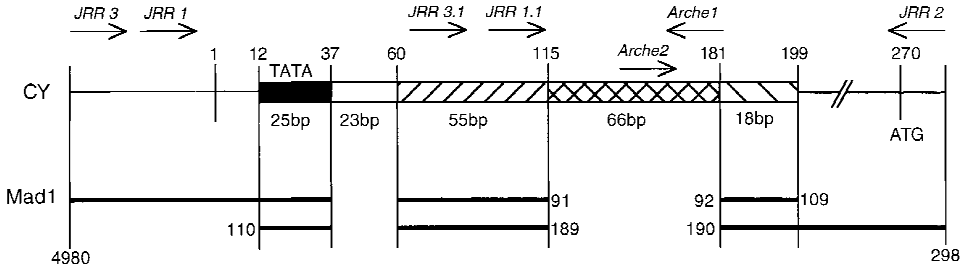

Fig. 1. Annealing sites of PCR primers and hybridization probes within the archetype (CY) and rearranged (Mad 1) TCRs. Table I lists the nucleotide sequences of the PCR primers and hybridization probes. The nucleotide numbering starting at position ≠1 in the archetype (CY) is that of Yogo et al. [1990]. Nucleotide numbering in Mad 1 is that of Frisque et al. [1984]. Mad 1 contains duplicate copies of a 25 bp region containing the TATA box, and a 55 bp and 18 bp region to yield a 98 bp tandem repeat. Mad 1 lacks the 23 bp and the 66 bp sequences found in archetype CY. Sequences encoding the early proteins large T, small t, and T′ are to the left of primer JRR3. The initiation codon for agnoprotein is within primer JRR2. VP1, VP2, and VP3 coding regions are to the right of JRR2.

light, and use of positive displacement pipettors (Gilson, Villiers–le–Bel, France). To monitor the possibility of contamination, a number of negative controls were included.

To determine the level of sensitivity of our PCR procedure, positive control reactions containing 10^1 to 10^6 molecules of recombinant JCV DNA were run in parallel with the tissue samples. These reactions were assembled after all negative controls and tissue-containing samples were sealed to avoid the possibility of cross–contamination. Positive signals were detected routinely with 10^1 or 10^2 input molecules; some variation in sensitivity was observed depending upon the primer pair utilized in the reaction.

PCR Conditions

Each PCR reaction was performed in a 50 μl reaction mixture containing thermophilic buffer (50 mM KCl, 10 mM Tris-HCl [pH 9.0], 0.1% [vol/vol] Triton X-100), 1.5 mM $MgCl_2$, 2.5 U of Taq DNA polymerase, 200 μM each of dATP, dCTP, dGTP, and dTTP (Pharmacia Biotech, Uppsala, Sweden), and one of the following primer pairs: JRR1 and JRR2 (0.5 μM each), JRR2 and JRR3 (0.5 μM each), Arche1 and JRR1 (0.6 μM and 0.4 μM respectively), or Arche2 and JRR2 (0.5 μM each) (Table I and Fig. 1). Most reactions were overlaid with 40 μl of mineral oil; in some reactions, the mineral oil and $MgCl_2$ were replaced with a HotWax Mg^{2+} bead (Invitrogen, San Diego, CA) containing $MgCl_2$ (final concentration 1.5 mM) to enhance the signal and reduce non-specific priming. Reactions were amplified in a Perkin-Elmer Cetus DNA Thermal Cycler through 40 cycles as previously described [White et al., 1992]. A preheating step (2 min, 94°C) was included in the cycling conditions when utilizing the HotStart beads. Positive controls contained 10^1 to 10^6 input molecules of recombinant prototype JCV DNA, pM1TCR1A [Frisque et al., 1984], when primer pairs JRR1/JRR2 or JRR2/JRR3 were used, and the same amounts of recombinant archetype JCV DNA, pJC-CY [Yogo et al., 1990], when primer pairs JRR1/Arche1 or Arche2/JRR2 were used.

Gel Electrophoresis and Southern Blotting

PCR products were electrophoresed on composite gels containing 0.7% molecular biology certified agarose (Eastman Kodak, Rochester, NY), 2% NuSieve GTG agarose (FMC BioProducts, Rockland, ME), 1X TBE buffer, and 0.5 μg/ml ethidium bromide. After electrophoresis, DNA was transferred from the gels onto Hybond N+ membranes (Amersham, Arlington Heights, IL) as previously described [White et al., 1992] with the exception that blotting was done in 6X SSC. After blotting, DNA was crosslinked to the membrane using a UV–crosslinker delivering 120,000 μJ/cm^2 at 254 nm wavelength for 5 min. Oligonucleotides JRR1.1 or JRR3.1 were used as hybridization probes to detect PCR products generated by primer pair JRR1/JRR2. JRR1 was used as a hybridization probe when primer pair JRR2/JRR3 was used. Conditions for prehybridization, labelling, hybridization, and washing were described previously [White et al., 1992]. Membranes were exposed to X–OMAT AR film (Eastman Kodak, Rochester, NY) for 1 to 3 hr.

DNA Cloning and Isolation

PCR products generated by primer pairs JRR1/JRR2 and JRR2/JRR3 were cloned into the TA cloning vector pCRII (Invitrogen), and products generated with the primer pairs JRR1/Arche1 and Arche2/JRR2 were cloned into the TA cloning vector pCRII 2.1 (Invitrogen, La Jolla, CA). Ligation and transformation reactions were carried out as described in the TA–Cloning Kit. Recombinant DNA was isolated from selected ampicillin-resistant transformants using the Wizard Plus Minipreps Kit (Promega, Madison, WI) and digested with *BstXI* and/or *AflIII* to identify clones containing JCV DNA. Mad6 is a full length viral genome that was previously cloned from virus following inoculation of cultured cells with extracts of this patient's brain tissue and was generously provided by Dr. Jonathan Martin (Mercer University School of Medicine).

DNA Sequencing

Recombinant DNAs which contained an appropriately-sized insert were sequenced manually by the Sanger dideoxy method [Sanger et al., 1977] or automatically with fluorescent terminators using an ABI 377 Prism Sequencer (Perkin–Elmer, Oak Brook, IL). The latter reactions were performed by the Nucleic Acid Facility at the Penn State University Biotechnology Institute. In the manual method, reactions were conducted at 37°C using primers PCRII#1, PCRII#2, M6R#1, and M6R#2 (Table I), 20 μCi α-[^{32}P]dATP (New England Nuclear, Boston, MA), and 1.5 μl sequencing grade Klenow enzyme (1 U/μl). Reaction products were electrophoresed on 7% or 8% denaturing polyacrylamide gels for 4 or 2 hr, respectively. Gels were exposed for 24 to 36 hr to BioMax film (Eastman Kodak).

RESULTS

Detection of JCV DNA in Multiple Tissues From a Pediatric PML Patient

Most PML patients are thought to have been initially infected with JCV early in life [Padgett and Walker, 1973; Taguchi et al., 1982; Walker and Padgett, 1983]. The onset of PML in later years is believed to result from the reactivation of this persistent infection during a prolonged immunocompromising condition [Frisque and White, 1992; Tornatore et al., 1994]. The unusually young age of PML onset in the present case suggests that the disease may have arisen during a primary infection. In a previous study, JCV DNA was detected in several tissues from this patient using Southern blot analysis [Grinnell et al., 1983]. However, no attempt was made to isolate or characterize the viral DNA.

With the advent of PCR technology, characterization of the JCV DNA present in these tissues became possible. We designed primer pairs that would allow us to examine the viral regulatory sequences present, since these sequences are known to undergo rearrangement in PML patients. The nucleotide sequences for the primer pairs used in the PCR reactions are shown in Table I, and their annealing sites within the viral noncoding region are shown in Figure 1. Since these primer pairs were designed to amplify both the archetypal and rearranged forms of JCV, our approach would allow us to investigate the form(s) of JCV present, the distribution of virus in the body, and the possibility that different forms might be associated with different tissues.

PCR was conducted on eight different tissues, and the products were electrophoresed on composite 0.7% agarose–2% NuSieve gels (Fig. 2a). In all tissues tested, amplification with either primer pair JRR1/JRR2 or JRR2/JRR3 produced one or more bands (Fig. 2a and data not shown).The two major bands detected in cardiac muscle (Fig. 2, lane Cm) correspond in size to the two different clones obtained from that tissue (Cm10, 273 bp, and Cm14, 328 bp; Fig. 3). We noted that some of the bands representing DNA amplified from the other tissues migrated more slowly than expected (Fig. 2a) given our sequence data (Fig. 3). Retarded migration of these DNAs might be attributed to differences in the compositions of the tissue extracts. To confirm the identity of the DNA detected in the PCR reactions, products were blotted onto a nylon membrane and probed with the appropriate oligonucleotide (Fig. 2b and data not shown). In control reactions, 10 input JCV genomes could be detected routinely (data not shown).

Identification of Archetype and Rearranged Forms of JCV in Multiple Tissues

To identify the type of JCV DNA detected in the various tissues, amplification products were cloned into the TA Cloning kit vector pCRII (Invitrogen). Recombinant DNA was prepared from the resulting transformants and digested with restriction enzymes *BstXI* and/or *AflIII* to release the viral DNA insert. Clones containing the appropriately-sized DNA fragments were subjected to sequence analysis using primers PCRII#1, PCRII#2, M6R#1, and M6R#2 (Table I). The results are presented in Figure 3.

Kidney clones Kd2, Kd21, and Kd24 represent examples of archetype JCV. Each regulatory region has a single copy of the promoter-enhancer which contains a 23 bp and a 66 bp region; one or both of these regions are usually absent in rearranged types. In previous studies, several archetype isolates were cloned from urine and kidney, and variability at a number of nucleotide positions within their noncoding regions were noted [Agostini et al., 1995, 1996; Flægstad et al., 1991; Guo et al., 1996; Yogo et al., 1990]. Using the archetype reference strain CY [Yogo et al., 1990] for comparison, clone Kd2 is identical at each of these positions except at nucleotide #217; clone Kd2 has an A, and CY has a G. Since this transition has been characterized in other isolates cloned directly from urine [Yogo et al., 1990], it is unlikely to have been introduced by Taq polymerase. In addition, an A occurs at this position in all DNAs cloned from this patient. Additional alterations observed in some clones obtained from each of the tissues examined in this study are shown in Table II.

Archetype-like clones were also isolated from cardiac muscle (Cm10, Cm14, Cm22) and lung (Lu33). These regulatory regions include only one copy of the promoter-enhancer, the 23 bp region, and except for Cm10, the 66 bp region. Nearly all archetype or archetype–like strains isolated previously were derived from the urine and kidneys [Agostini et al., 1995, 1996; Chang et al., 1996; Guo et al., 1996; Kitamura et al., 1994a,b; Loeber and Dörries, 1988; Markowitz et al., 1991; Yogo et al., 1990, 1991b]; rare examples of archetype-like JCV from diseased brain have been reported [Ault and Stoner, 1993].

Multiple clones were obtained from the other tissues available from this patient, and all were shown by se-

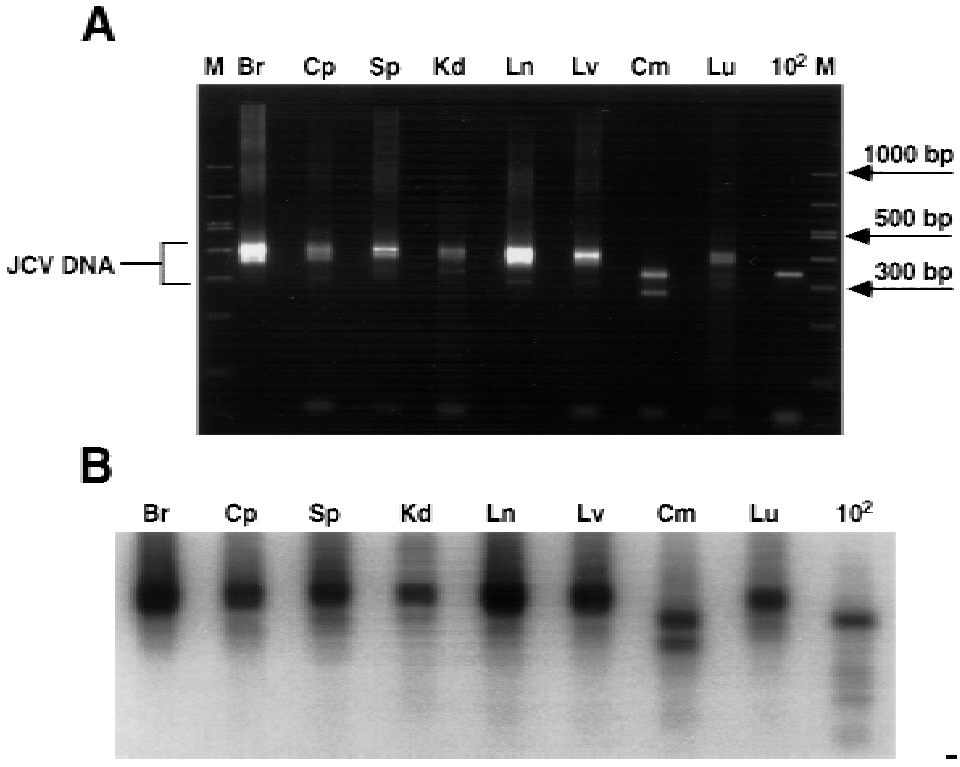

Fig. 2. Detection of JCV DNA in the tissues obtained from a pediatric case of PML. **A**: PCR products amplified from different tissues using primer pair JRR1/JRR2 were electrophoresed on a composite 2% NuSieve–0.7% agarose gel. **B**: Southern blot of the gel in (A with oligonucleotide JRR3.1 used as a hybridization probe. Abbreviations from left to right are: Br, brain; Cp, coeliac plexus; Kd, kidney; SP, spleen; Lv, liver; Ln, lymph node; Lu, lung; Cm, cardiac muscle; M, marker. The 10^2 lane contained the amplified PCR product of 100 input molecules of recombinant prototype JCV DNA, pM1TCR1A [Frisque et al., 1984], used as a positive control. The position of JCV DNA on the gel is indicated by a bracket, and the sizes of selected marker bands are shown in base pairs (bp).

quence analysis to differ from archetype JCV, being characterized by deletions and two, three, or four tandem repeats within their promoter-enhancer regions. Furthermore, clones obtained from the same tissue often differed from each other in their sequence. For example, Br9, Br10, Br11, and Mad6 had distinct TCRs (Fig. 3), and are different from three additional clones from the brain of this patient [Ault and Stoner, 1993]. Some of the JCV clones detected in the brain were identified subsequently in other tissues. Clone Br9 was also amplified and cloned from lymph node (Ln2, Ln7), liver (Lv11), and coeliac plexus (Cp1) (Fig. 3 and Table II). One clone isolated from brain and lung tissue (Br11 and Lu31, respectively) had a unique structure; it represents the first rearranged form of JCV found to have two TATA boxes and an intact 23 bp region. The only other strain of JCV that contains a duplicated TATA box within its promoter-enhancer is Mad 1, and it lacks the 23 bp sequence [Frisque et al., 1984]. Not all rearranged clones isolated in our study were detected in brain. Clone Cp3 from coeliac plexus and clones Sp6 and Sp19 from spleen were not found in brain. These clones were essentially identical, and were clearly related to Ln3, Ln9, Lu20, Lv18, and Lv31 which contain two rather than three copies of the promoter–enhancer element.

Detection of JCV DNA in Brain and Lymph Node Tissue Using Archetype–Specific Primers

The amplification of archetype-like JCV TCR sequences from cardiac muscle and lung demonstrated that this form of JCV was not confined to the kidney. To increase the probability of detecting archetype JCV in some of our previously-tested tissues, the primer pair JRR1/Arche1 was employed. Arche1 anneals to the 66 bp region (Fig. 1) which is absent in most rearranged forms of JCV. The results of a PCR reaction conducted using brain and lymph node tissues are shown in Figure 4. In both tissues, a fragment having the expected size (230 bp) for amplified archetype sequences was detected. PCR products were cloned and two clones from brain (Br13, Br14) and one from lymph node (Ln20) were sequenced; all three viral regulatory regions contained both the 23 bp and the 66 bp regions and a single copy of the promoter-enhancer (Fig. 5). Clone Br13 contained two base pair changes relative to clone Kd2 (Table II).

One additional PCR reaction was conducted with a second set of archetype–specific primers (Arche2/JRR2) which would allow us to examine sequences to the late side of the 66 bp region. Because the clones

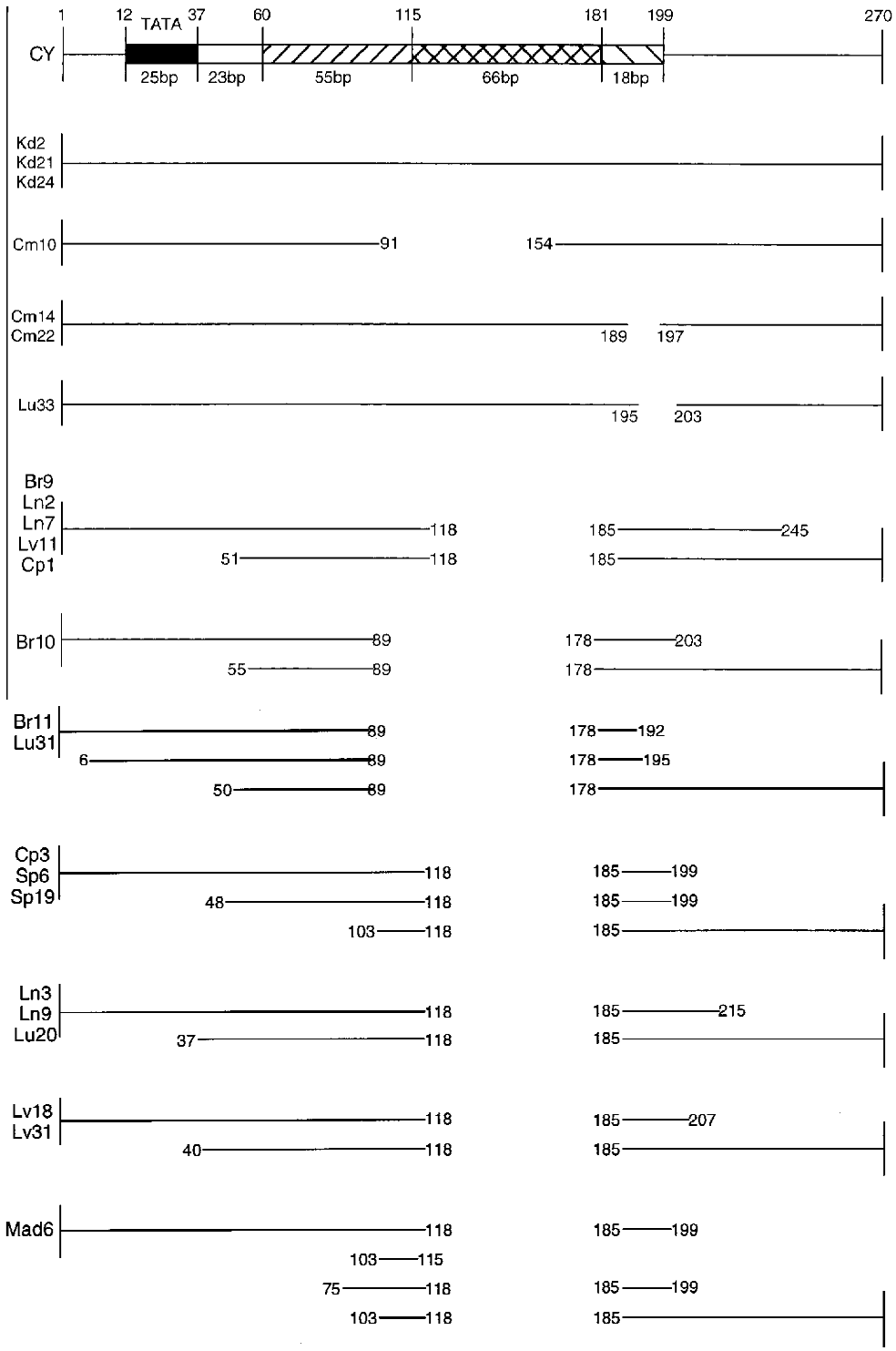

Fig. 3. Structures of the JCV TCRs derived from multiple tissues of a pediatric PML patient. Nucleotide numbers represent the positions within the archetype CY sequence [Yogo et al., 1990]. Clones are identified at the left and are followed by a line which is read from left to right. A gap within a line represents a deletion of archetype sequence, and parallel lines represent multiple copies of that particular sequence. Abbreviations for tissues are identified in the legend for Figure 2. Clone Mad6 is a full length genome cloned from this patient previously (Dr. Jonathan Martin, Mercer University School of Medicine).

Cm14, Cm22, and Lu33 (Fig. 3) contained small deletions in this region, the Arche2/JRR2 primer pair was used to distinguish between archetype and archetype–like sequences amplified from brain and lymph node. PCR analysis of both tissues produced products of about 150 bp in size (Fig. 4). Sequence analyses of three clones obtained from brain (Br15, Br16, and Br17; Fig. 5) were identical to the archetype clone Kd2 (Fig. 5).

TABLE II. Nucleotide Changes Within the Promoter-Enhancer of JCV Clones Relative to the Archetype CY Strain

Clone[a]	Alteration	Position (PE copy#)[b]	Source of variation[c]
All[d] (30/30)	G→A	217	natural
Kd21 (1/3)	T→C	225	PCR
Cm10 (1/3)	A→G	45	PCR
Cm14 (1/3)	T→C, C→T	140, 162	PCR
Cm22 (1/3)	ΔA, A→G	29[e], 33	PCR
Br9 (1/3), Ln2 (2/4), Ln7 (2/4), Lv11 (1/3), Cp1 (1/2)	ΔA	56[f] (#2)	natural
Ln2 (1/4)	ΔA	29[e] (#1)	PCR
Ln3 (1/4)	T→C	252 (#2)	PCR
Ln3 (2/4), Ln9 (2/4), Lu22 (1/3)	T→C	37 (#2)	natural
Lu31 (1/3)	C→T	51 (#3)	PCR
Lv31 (1/3)	A→T	74 (#2)	PCR
Cp3 (1/2)	A→G	115 (#3)	PCR
Sp19 (1/2)	T→C, A→G, A→G	76, 70 (#2), 108 (#3)	PCR
Mad 6	ΔG	41[g] (#1)	natural
Br13 (1/2)	A→G, A→G	56, 156	PCR
Ln21 (1/4)	ΔG	#211	PCR

[a]The ratio in parenthesis represents the number of clones obtained from a specific tissue and which contained the alteration divided by the total number of clones derived from that tissue. Most clones were amplified using primer pair JRR1/JRR2 or JRR2/JRR3; clones Br13 and Ln21 were obtained using primer pairs JRR1/Arche1 and JRR2/Arche2 respectively, and Mad6 was cloned directly from isolated virus.
[b]Nucleotide numbering is from Yogo et al. [1990] for the CY archetype strain. "PE copy#" identifies the copy of the promoter-enhancer which contains the alteration.
[c]"Source of variation" indicates whether the alteration was predicted to be the result of natural variation or an error introduced by Taq polymerase.
[d]"All" refers to the total number of clones obtained from the eight tissues using primer pairs JRR1/JRR2, JRR2/JRR3, or JRR2/Arche2.
[e]The deleted A occurs within a stretch of eight A residues; it could represent any A between position 22 and 29.
[f]The deleted A occurs within a stretch of four A residues; it could represent any A between position 53 and 56.
[g]The deleted G occurs within a stretch of three G residues; it could represent any G between position 39 and 41.

One clone obtained from lymph node (Ln 21) was identical to clone Kd2 except for a deletion of a G residue at position #211 (Fig. 5 and Table II). A second clone, Ln25 was identical to Cm14 and Cm22 over the region sequenced (compare Fig. 3 and Fig. 5). Two other clones obtained from this tissue (Ln 23 and Ln24) contained small deletions similar to those detected in Cm14, Cm22, and Lu33 (Compare Fig. 3 and Fig. 5). All sequenced clones contained an A residue at the position corresponding to nucleotide #217 of CY, confirming that our results with the archetype–specific primers were not due to the contamination of the samples with the CY strain of archetype. The specificity of primer pairs JRR1/Arche1 and Arche2/JRR2 was confirmed in reactions utilizing 10^6 input molecules of Mad 1 and clone Br10 as templates; no amplification of these DNAs was observed (data not shown).

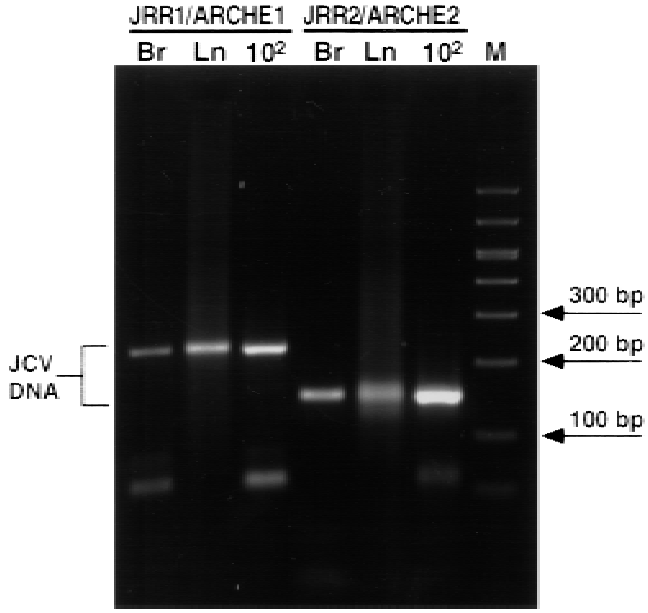

Fig. 4. Detection of archetype DNA in brain and lymph node tissues using archetype-specific primer pairs. PCR products amplified from brain and lymph node tissues using primer pair JRR1/Arche1 or Arche2/JRR2 (Fig. 1) were loaded onto a composite 2% NuSieve–0.7% agarose gel. Abbreviations for each lane are as follows: Br, brain; Ln, lymph node; 10^2, 100 input molecules of recombinant pJC-CY [Yogo et al., 1990]); M, marker. Primer pairs used are indicated above each lane. The positions of JCV DNA on the gel is indicated by a bracket, and the sizes of selected marker bands are shown in base pairs (bp).

DISCUSSION

JCV DNA has been detected in the tissues and body fluids of immunocompetent and immunosuppressed patients [Tornatore et al., 1994]. Based upon the structure of the viral TCR, two types of JCV have been identified; the archetypal form which has been found in kidney and urine [Flægstad et al., 1991; Guo et al., 1996; Loeber and Dörries, 1988; Yogo et al., 1990], and a group of rearranged forms which have been detected in the brain, kidney, and lymphocytes [Loeber and Dörries, 1988; Martin et al., 1985; Tornatore et al., 1992; White et al., 1992]. It has been suggested that the archetypal form is transmitted from an infected individual to a susceptible host, and the rearranged forms are then generated within a newly-infected person [Ault and Stoner, 1993; Flægstad et al., 1991; Yogo et al., 1991a,b]. Currently, there is very little information available regarding where and when this rearrangement occurs in the body or which factors contribute to the process. The findings reported in this study are relevant to the search for answers to these important questions.

We have amplified JCV DNA from the multiple tissues of a young PML patient using PCR, and for the first time have characterized the TCR of the viral DNA present at sites other than brain, kidney, and lymphocytes. Because of the potential sequence heterogeneity of the viral TCRs amplified from the tissues, PCR products were not sequenced directly, but instead were

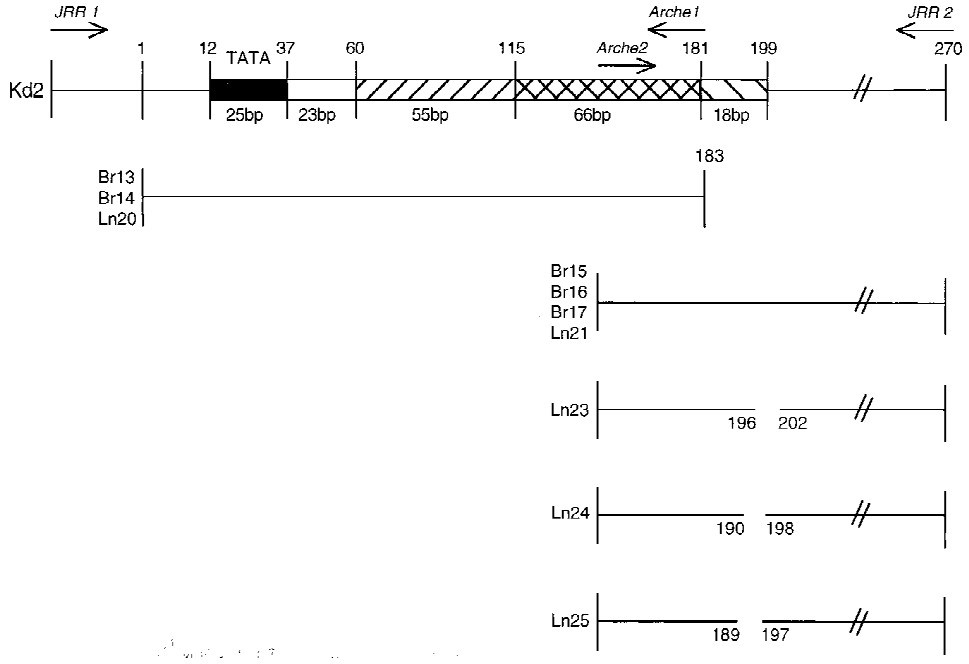

Fig. 5. Structures of JCV TCRs derived from brain and lymph node using archetype–specific primers. Nucleotide numbers represent positions within clone Kd2. Clones are identified at the left and are followed by a line which is read from left to right. Abbreviations are: Br, brain; Ln, Lymph node. The positions of primer pairs JRR1/Arche1 and Arche2/JRR2 used to obtain clones Br13, Br14, Ln20 and clones Br15, Br16, Br17, Ln21, Ln22, Ln23, Ln24, Ln25, respectively, are shown above clone Kd2.

cloned to permit analysis of individual recombinant DNAs.

PCR primer pairs capable of recognizing both archetype and rearranged JCV directed the amplification of archetype and archetype-like variants from kidney, cardiac muscle, and lung, and rearranged forms from lung and five other tissues. Given the limited number of clones sequenced, it is likely that our approach identified the major species of TCR present in a particular tissue. This in turn might suggest that archetype and rearranged JCV have different replication potentials in the various tissues. It is important to note that when archetype–specific primer pairs were used, archetype and archetype–like variants were detected in brain and lymph node respectively, thus indicating that this form of JCV does reach sites other than the kidney but is likely to be present at low levels.

JCV genomes isolated from different PML patients nearly always have a uniquely rearranged TCR, but within most individuals, only a single variant is found [Ault and Stoner, 1993; White et al., 1992]. An exception to the latter observation was reported by Yogo et al. [1994], who cloned several different variants from the brain of a 33-year-old PML patient with AIDS. Two major groups of variants were recognized based upon the location of the deletion endpoints in the rearranged TCRs. In the present study of the pediatric PML patient, multiple rearranged JCV variants were also identified. Although even greater variability was seen here, it again appears that most of the variant TCRs arose after one of two deletion events affecting sequences between nucleotides 118 and 185 or 89 and 178 (Fig. 3). Some of the deletion boundaries observed are the same as those seen in variants isolated from other PML patients [Kato et al., 1994]. Following the deletion event, a variable number of duplication events took place to yield two to four tandem repeats within the viral TCR. As discussed below, it is possible that the presence of multiple JCV variants in the 5-year-old SCID and 33-year-old AIDS patients might be a result of their immune status at the time of exposure to the virus.

In addition to the sequence diversity generated by rearrangement of the JCV TCR, different strains of the virus have also arisen in people inhabiting different geographic areas. These strains are distinguished from one another primarily by single nucleotide changes scattered throughout the genome [Agostini et al., 1995, 1996; Ault and Stoner, 1992; Guo et al., 1996; Iida et al., 1993; Kunitake et al., 1995; Tominaga et al., 1992; Yogo et al., 1990, 1991a]. In the present report, nucleotide variations (relative to the archetype CY TCR sequence) were detected in many of the clones (Table II). It is likely that most of these changes represent errors introduced by Taq polymerase since: 1) Alterations are randomly distributed; 2) in most cases, these changes do not correspond to previously documented strain variations; and 3) the error frequency observed is within the range reported for this enzyme [Cline et al., 1996]. However, some alterations are probably not due to enzyme infidelity. The G to A transition at position #217 was found in all of our clones indicating that this was an authentic change. This finding also supports the idea that the patient was infected with a single

archetype strain from which all of the rearranged variants were derived. In addition, we also detected two single nucleotide changes which may have been generated during the course of the infection. A missing A residue at position #56 in the second copy of the promoter-enhancer of clones Br9, Ln2, Ln7, Lv11, Cp1, and a T to C transition in clones Ln3, Ln9, and Lu22 (Table II) may have arisen during the rearrangement process since both changes occur at a deletion boundary.

While our analysis revealed that multiple variants were present in a single tissue, we also observed that a particular variant could be found in multiple tissues; clone Br9 from brain was also isolated from lymph node, liver, and coeliac plexus. These observations may be relevant to the questions of where and when these sequence rearrangements occur. Although the initial site of JCV infection has not been determined, it is possible that upon entry of archetype into the host, the virus infects lymphoid tissue and replicates. If rearrangement of the TCR takes place during active viral replication in lymphocytes at this site, these cells might then transport archetype and rearranged JCV throughout the body. Some of these possibilities have already been discussed by others based upon detection of JCV in the lymphocytes of immunosuppressed individuals and PML patients [Dörries et al., 1994; Tornatore et al., 1992]. It is also possible that the rearrangement occurs in non–lymphoid tissue and that lymphocytes simply seed the virus to other tissues. In either case, once present at specific secondary sites, the two forms of JCV would likely differ in their ability to establish a focal infection, and thus one of the two forms would become predominant in that tissue.

In most cases, PML probably results from reactivation of a persistent infection established during the first two decades of life [Frisque and White, 1992]. However, in the present study, PML likely arose as a consequence of a primary infection in a child with underlying combined immune deficiency. These differences in patient profiles may have contributed to the more extensive rearrangement and distribution of the virus observed in the pediatric patient. In an immunocompetent individual, only a limited number of variants may become established in the body prior to control of the primary infection by a functioning immune system. Later, if PML does occur during a severe immunocompromising event, only the predominant variant(s) might be detected. On the other hand, in the absence of an effective immune response, as with the 5-year-old child and perhaps the 33-year-old AIDS patient described earlier [Yogo et al., 1994], viral replication may proceed unchallenged after the primary infection. Presumably, rearrangement of the TCR and spread of the virus to multiple sites would be enhanced under these conditions. In the future, it will be important to analyze reactivation cases of PML and to reevaluate the extent of rearrangement and distribution of JCV in such an individual.

ACKNOWLEDGMENTS

We thank D. Walker for providing tissue specimens and J. Martin for recombinant DNA Mad6. We would also like to thank the members of our laboratory for their helpful suggestions during the preparation of this manuscript. This work was supported by Public Health Service Grant CA44970 from the National Cancer Institute. JTN was supported in part by a Sigma Xi Grant–in–Aid of Research.

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